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B lymphocyte lineage-restricted expression of *mb-1*, a gene with CD3-like structural properties

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A gene, called m-*mb-1*, was isolated from a murine pre-B-minus T lymphocyte subtracted library. It was found expressed as mRNA at low to medium abundance in early progenitors of the B lineage, in pre-B and mature B lineage cell lines, in normal resting B lymphocytes and in polyclonally activated B cell blasts. The gene was not expressed in plasmacytomas, in cell lines of the monocyte/macrophage, the T lymphocyte or the fibroblast lineages, nor in thymus, liver, heart, kidney, lung or brain. The nucleotide sequence of the m-*mb-1* gene encodes a putative membrane glycoprotein with 220 amino acids, which includes a leader sequence, a putative extracellular domain with two potential N-glycosylation sites, a transmembrane portion and a putative intracellular domain. The partial sequence of a human homologue, h-*mb-1*, shows nearly 90% homology in nucleotide as well as amino acid sequences to the murine form of a stretch of the putative intracytoplasmic domain. Antibodies raised against a fusion protein of m-*mb-1* with protein A, affinity purified for their m-*mb-1* specificity, stained pre-B and mature B cell lines on their surface, but did not stain T cell lines and fibroblasts. Antibodies raised against a stretch of 20 amino acids of the putative intracellular domain with 90% homology between the mouse and human protein did not stain the surface of any cell lines tested. However, they stained the cytoplasm of pre-B and B cell lines, but not of plasmacytomas, T cell lines or fibroblasts. *mb-1* is discussed as a potential member of a CD3-like complex on cells of the B lineage, since the overall structure of the deduced protein and the homologies between the murine and the human form show striking similarities to the γ and δ chains of the CD3 complex expressed on T cells.

Key words: gene *mb-1*/CD3 complex/B cells

Introduction

The development of the B lymphocyte lineage from stem cells and early progenitors to mature B cells and immunoglobulin (Ig)-secreting plasma cells is one of the best-documented pathways of eukaryotic differentiation. Steps in this development have been characterized by the genomic context in which Ig gene segments for heavy (H) and light (L) chain genes are found, by the state of Ig expression, by surface markers and by susceptibilities to growth and differentiation factors (Tonegawa, 1983; Melchers *et al.*, 1977; Kincade *et al.*, 1981; Coffmann and Weissman, 1981; McKearn *et al.*, 1984; Cooper *et al.*, 1986). A series of transformed cell lines and tumours exist which represent various stages of B cell differentiation in a 'frozen' form. We have begun a search for genes which are selectively expressed at certain stages of B cell development with the aim of developing a set of new markers and to find gene products which control this developmental pathway. Two genes, λ_5 (Sakaguchi *et al.*, 1986; Sakaguchi and Melchers, 1986; Kudo *et al.*, 1987) and $V_{preB}1$ (Kudo and Melchers, 1987) have been identified which are expressed in pre-B cells only. Here we report the structure and pattern of expression of a new gene, m-*mb-1*, which is selectively expressed at the pre-B and B cell stages of B cell development in the mouse, and which may be a member of a CD3-like complex on cells of the B lineage.

Results

Isolation of cDNA clones which are selectively expressed in B lineage cells

We used a pre-B cell-selected cDNA library which we had constructed previously (Sakaguchi *et al.*, 1986) and from which we had isolated the pre-B cell-specific cDNA clone pZ183 encoding the λ_5 gene (Sakaguchi and Melchers, 1986) to search for other genes which might be selectively expressed in B lineage cells. In the initial screening we selected 200 clones which hybridized with cDNAs from the pre-B lymphoma 70Z/3 but not with cDNA from the T hybridoma K62 (Sakaguchi *et al.*, 1986). From these 200 cDNA clones, individual clones were tested for expression on a panel of RNA samples from various cell lines including lines of pre-B cells, mature B cells, T cells, monocytic cells and fibroblasts. These cell lines are thought to be 'frozen' at a certain stage of normal development and, thus, are considered to represent the phenotype of the normal counterpart. The panel also included an early progenitor cell line, HAFTL-1 (Holmes *et al.*, 1986), which can give rise to subclones with either monocytic (clone 2B7) or pre-B cell characteristics (clone 6). One of these cDNA clones, named m-*mb-1* pZ176, was selected.

Results in Figure 1 show that m-*mb-1* is expressed only in cell lines of the B lineage and in the early progenitor cell line HAFTL-1. Expression is active in pre-B cell lines from the early stages of D_HJ_H rearrangements (shown in Figure 1 is the cell line 204-1-8) to the late stages of productive $V_HD_HJ_H$ and V_LJ_L rearrangements (shown in Figure 2 is the cell line 70Z/3), continues in the surface Ig-positive, MHC class II-expressing mature B cells and is turned off when monocytes (HAFTL-1 subclone 2B7) or when plasma cells develop. A more detailed description of the properties of the cell lines used in the experiments shown in Figure 1 is given in a previous publication (Sakaguchi *et al.*, 1986). The collection of pre-B cell lines, B cell lines and plasma cell

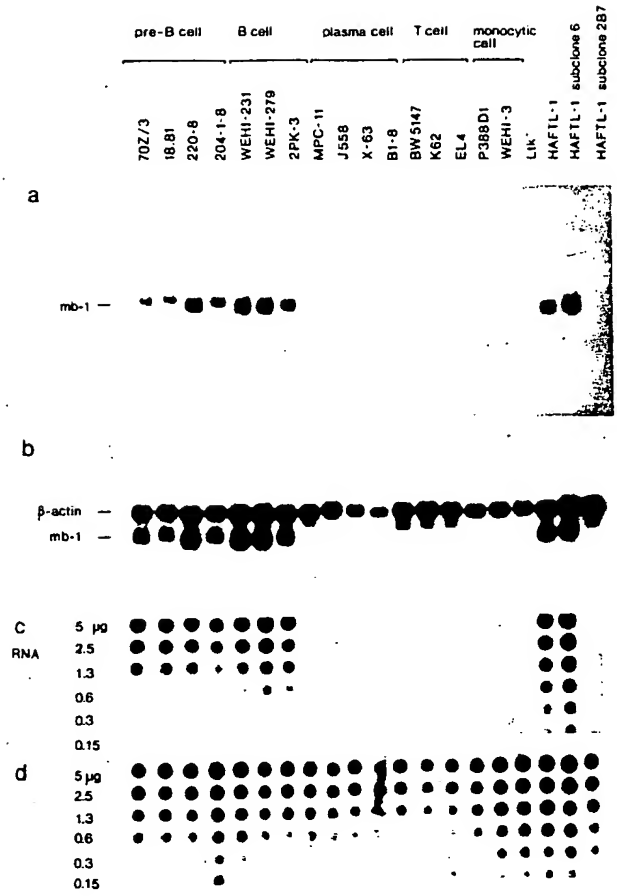


Fig. 1. Expression of m-*mb-1* transcripts in eukaryotic cell lines of different stages and lineages, and quantitative dot-blot analysis of m-*mb-1* transcripts in cell lines. Cytoplasmic poly(A)$^+$ RNA (5 µg) from various cell lines was subjected to electrophoresis in 1.0% agarose/formaldehyde, transferred to nylon membrane and hybridized with the radioactive probe of m-*mb-1* (**a**) or β-actin (**b**). Several dilutions of cytoplasmic total RNA from various cell lines were dotted onto nitrocellulose filter for hybridization with the probe of m-*mb-1* (**c**) or β-actin (**d**).

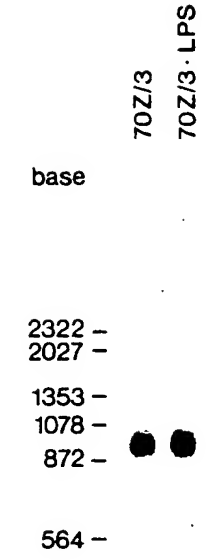


Fig. 2. Size determination of the m-*mb-1* mRNA by Northern blot analysis of RNA isolated from non-stimulated and LPS-stimulated 70Z/3 cells. Cytoplasmic poly(A)$^+$ RNA (5 µg/lane) was isolated from 70Z/3 cells cultured with (**b**) or without (**a**) LPS (10 µg/ml) for 12 h.

lines tested for m-*mb-1* expression is larger than that shown in Figure 1. In all these additional cases m-*mb-1* expression followed the rule that it is on in pre-B and mature B cells, and off in plasma cells.

The size of m-*mb-1* transcripts was analysed on Northern blots with a set of markers. RNA from unstimulated 70Z/3 as well as from lipopolysaccharide (LPS)-stimulated 70Z/3 cells was found to be ~1 kb in length (Figure 2). Results in Figure 1a and b show that m-*mb-1* RNA had the same size in all pre-B and B cell lines tested.

The frequency of m-*mb-1*-positive cDNA clones was determined as 1 in 180 in the B cell-selected cDNA library, 1 in 5000 in the unselected library from 70Z/3 pre-B lymphoma cells and also 1 in 5000 in an unselected library from the mature B lymphoma WEHI 231. This indicates that the gene is expressed at low to medium abundance throughout pre-B and B cell development.

The level of m-*mb-1* RNA expression in pre-B and B cell lines could also be estimated by RNA dot-blot analysis of serially diluted RNA samples from the different cell lines (Figure 1c and d). If plasma cells, T cells, monocytic cells or fibroblasts express m-*mb-1* at all, it must be 10–50 times lower than expression in the positive pre-B and B cell lines. Expression of m-*mb-1* in all positive cell lines, i.e. along the B cell differentiation pathway, appears to be similarly high.

Expression of the m-mb-1 gene in normal tissues and cells

Poly(A)$^+$ RNA prepared from a variety of tissues was tested for expression of the m-*mb-1* gene. Thymus, liver, kidney, heart, lung and brain did not express m-*mb-1* RNA in detectable quantities; spleen, bone marrow and fetal liver were weakly positive, again with an RNA species of ~1 kb in length (data not shown). Since m-*mb-1* had been found in all pre-B and B cell lines tested to be expressed at low to medium abundance, expression of m-*mb-1* in a minor population of cells in these organs may go undetected in preparations of total poly(A)$^+$ RNA from an organ. We therefore also tested m-*mb-1* RNA expression in single cells of fetal liver and thymus (day 16 of gestation) by *in situ* hybridization (Berger, 1986) using a radioactive single-stranded m-*mb-1* cDNA probe. The opposite strand of m-*mb-1* was used as negative control probe, while the probes for λ_5 (Sakaguchi and Melchers, 1986) and a probe for β-actin served as positive controls. 70Z/3 pre-B lymphoma cells, used as positive control cells, were stained to >90% by the m-*mb-1*, λ_5 and β-actin probes, while the opposite strand of m-*mb-1* did not stain. Fetal thymus cells were >90% positive with the β-actin probe, negative for λ_5 and the opposite strand for m-*mb-1*, and 1–2% positive with the m-*mb-1* probe. Fetal liver cells were >90% positive with the β-actin probe, negative with the opposite strand of

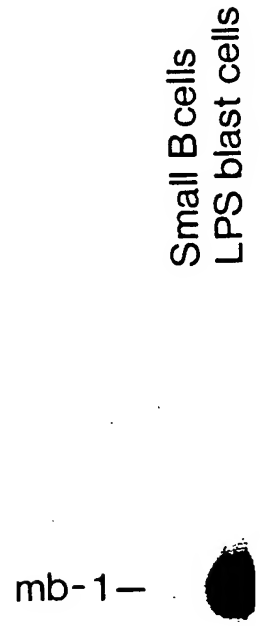


Fig. 3. Northern blot analysis of m-*mb-1* transcripts in cells of normal resting B cells and activated B cell blasts. Cytoplasmic poly(A)$^+$ RNA (5 μg/lane) from small resting B cells and from 3 day stimulated B blast cells were subjected to polyacrylamide gel electrophoresis, transferred to nylon membrane and hybridized with the radioactive probe. Blots were exposed to X-ray film for 7 days at −70°C in the presence of an intensifying screen.

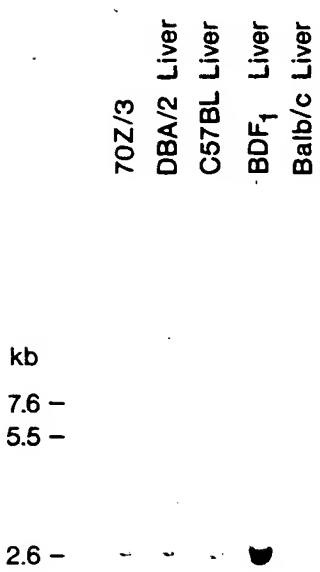


Fig. 4. Southern blot analysis of DNA from liver of DBA/2, C57BL/6J, (C57BL/6J × DBA/2J)F_1, BALB/c mice and from the 70Z/3 pre-B cell line. DNA was digested with *Eco*RI. The filter was hybridized with ^{32}P-labelled insert DNA of the cDNA clone m-*mb-1*-W-8.

m-*mb-1*, 1−2% positive with λ_5 and 5−15% positive with the m-*mb-1* probe.

Expression of m-mb-1 in normal resting and activated B lymphocytes

Up to this point of analysis it was conceivable that only proliferating mature B cell express the m-*mb-1* gene, since all transformed B cell lines were continuously growing. We therefore depleted C57BL/6J × DBA/2J mouse spleen cells of T cells and A cells and purified resting B lymphocytes by treatment with Thy1-specific antibody plus complement, followed by velocity sedimentation (for details see Materials and methods). The poly(A)$^+$ RNA prepared from these resting B cells was found to contain m-*mb-1* RNA (Figure 3). This indicates that the m-*mb-1* gene is expressed in normal, mature, resting B cells.

Plasmacytoma cells had been found not to express the m-*mb-1* gene (Figure 1). We therefore stimulated resting mature B cells with the polyclonal activator LPS (Andersson *et al.*, 1972) for 3 days, purified the resulting activated, dividing, Ig-secreting blast cells by velocity sedimentation and probed the poly(A)$^+$ RNA prepared from them by Northern analysis (Figure 3). It is evident that m-*mb-1* is expressed in 3-day activated, dividing, Ig-secreting B cells. *In situ* hybridization analysis of the number of B cell blasts activated *in vitro* for 3 days with LPS showed that >90% of them expressed RNA hybridizing with a probe for β-actin and a probe for sequences encoding a part of the constant region of μ-heavy chain, while the opposite strand of m-*mb-1* yielded no staining above background. The m-*mb-1* probe hybridized with a positive signal to at least 60% of all LPS-activated B cell blasts. We conclude that the majority of activated, dividing, Ig-secreting B cell blasts do express m-*mb-1*.

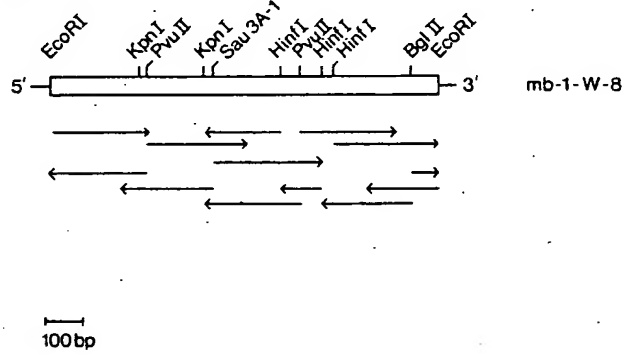
The context of the m-mb-1 gene within the murine genome

We tested the genome of 70Z/3 B lymphoma cells for a possible alteration in the arrangement of the m-*mb-1* gene by a restriction fragment length analysis of DNA from 70Z/3 cells in comparison to DNA from liver of C57BL/6 × DBA/2JF_1 (BDF_1) mice, from which the 70Z/3 lymphoma had originally been isolated, and to DNA from liver of the corresponding parental strains DBA/2J and C57BL/6J, as well as to DNA from liver of BALB/c mice. The data in Figure 4 indicate that the context of the m-*mb-1* gene is not altered by rearrangement, insertion or other modes of mutation which would be detectable as a change in the length of DNA fragments obtained by digestion of the DNA with *Eco*RI (shown in Figure 4), and also with *Hin*dIII and *Bam*HI (data not shown).

The nucleotide sequence of m-mb-1 cDNA and the deduced amino acid sequence

With the aid of the shorter original m-*mb-1* cDNA clone pZ176 a full length cDNA clone of m-*mb-1* was isolated from a library in λgt10 (Huynh *et al.*, 1985), prepared with

A



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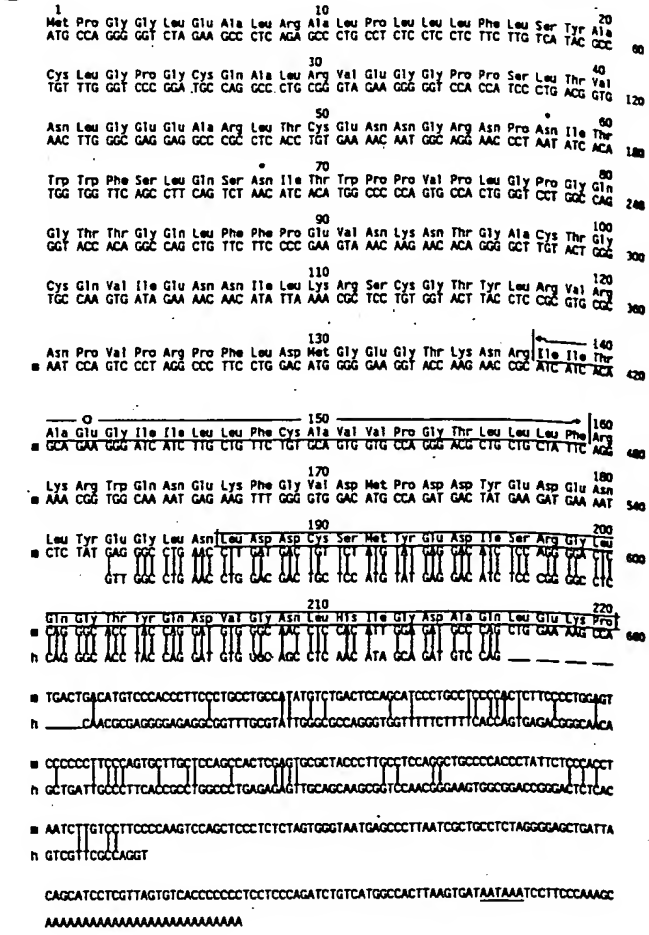

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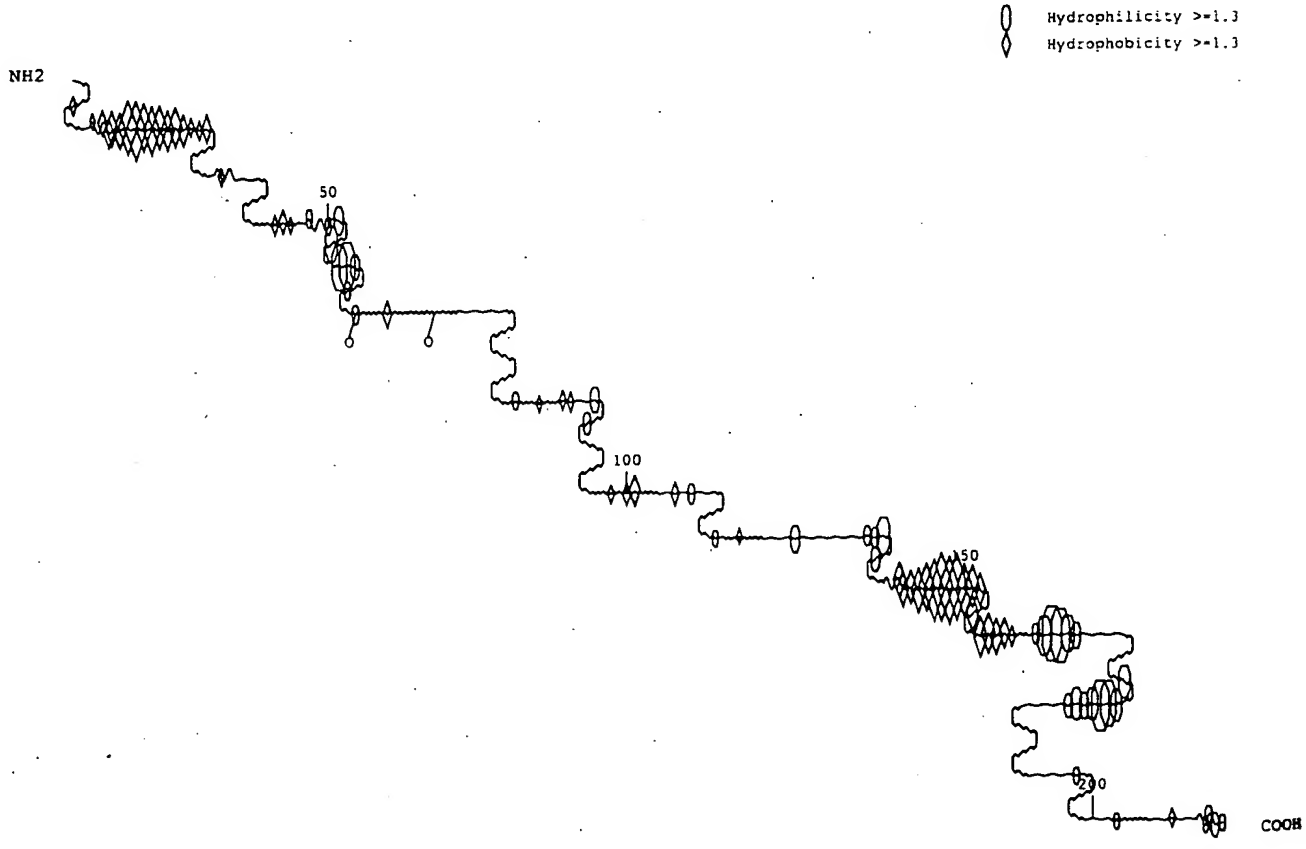

poly(A)$^+$ RNA from the surface Ig-positive B lymphoma WEHI 231 (Gubler and Hoffman, 1983). From a library of 3×10^5 clones, 60 were found positive for m-*mb-1* sequences. Six clones contained an insert of ~1 kb. They were further analysed by restriction enzyme mapping. One clone, m-*mb-1*-W-8, was then sequenced. The sequencing strategy, a detailed restriction map, the nucleotide sequence of the cDNA m-*mb-1*-W-8, the deduced amino acid sequence and the Chou–Fasman hydrophilicity/hydrophobicity plot (Chou and Fasman, 1978) of the amino acid sequence are shown in Figure 2a, b and c. A poly(A) tail and a consensus poly(A) attachment site allow the orientation of the cDNA sequence from 5′ to 3′. The longest open reading frame extends from an ATG at nucleotide position 1 to a TGA stop codon at nucleotide position 660. This sequence encodes a protein, m-MB-1, with 220 amino acids. There are two potential N-glycosylation sites at positions 58 (Asn-Ile-Thr) and 68 (Asn-Ile-Thr). A stretch of 22 hydrophobic amino acids from position 137 to 159 may serve as a transmembrane domain. Position 142 within this potential transmembrane domain contains a charged amino acid (Glu). m-*mb-1* also contains an NH_2-terminal leader sequence to position 25, which furthermore makes it a candidate for a gene encoding a membrane-bound protein expressed on pre-B and B cells. Because of the location of the two glycosylation sites we expect that the NH_2-terminal portion of m-MB-1, approximately two thirds of the whole protein, is the extracellular domain, while the COOH-terminal third of m-MB-1 should be the intracellular domain. A computer search in the Genbank nucleotide sequence data bases did not reveal any evolutionary relationship of m-*mb-1* to any known DNA sequences. We conclude from the structural analysis of the m-*mb-1* cDNA that the deduced protein encoded by this gene may be a membrane protein (MB-1) of 220 amino acids with a leader sequence, an extracellular, a transmembrane and an intracellular domain.

Partial sequence of a human homologue, h-mb-1

A cDNA library constructed from poly(A)$^+$ RNA of the human Burkitt lymphoma cell line Daudi was screened with a radiolabelled insert of the murine m-*mb-1*-W-8 clone as described in Materials and methods. One positive clone (of seven) was sequenced. As shown in Figure 5 the partial sequence (h) shows strong homology (~90% in nucleotide and amino acid sequences) to the murine m-*mb-1* gene.

m-mb-1 peptide- and fusion protein-specific antibodies and the cell surface and intracytoplasmic expression of m-mb-1 in pre-B and mature B cell lines

Two types of antibodies were raised in rabbits against potential antigenic determinants of the putative m-MB-1 protein encoded by the m-*mb-1* gene. First, a fusion protein between m-MB-1 and protein A was made by introducing cDNA inserts of the m-*mb-1*-W-8 clone into pR1T2T plasmid DNA containing the protein A gene, followed by expression in *Escherichia coli*. Chimeric protein purified over IgG–Sepharose was used as an immunogen in rats, and monoclonal antibodies were produced as described in Materials and methods.

Second, a peptide was synthesized which corresponds to the putative intracellular stretch from Leu at position 187 to Pro at position 220 (see Figure 5) (with ~90% homology between mouse and man). The peptide was coupled to ovalbumin, and used in this coupled form as an immunogen in rabbits. Peptide-specific antibodies were purified over peptide-coupled Sepharose as described in Materials and methods. The specificity of the antibodies was checked by enzyme-linked immunosorbent assays (ELISA) using protein A as control for the first type, peptides of other, m-*mb-1*-unrelated sequences, for the second type of antibodies.

Binding to the surface of a series of live pre-B, B, plasmacytoma, T, monocyte/macrophage and fibroblast cell lines (see panel in Figure 1) was tested by immunofluorescence staining and flow-fluorocytometry. Four examples are shown in Figure 6 for the first type of antibodies (the monoclonal antibody 1-20) specific for m-MB-1 as a fusion protein on protein A. Pre-B cell lines (shown in Figure 6 are 70Z/3 and 40E-1) and B cell lines (WEHI 231, WEHI 279, not shown) were positive for staining on the surface, while plasmacytomas (P_3U, shown in Figure 6), T hybridomas and lymphomas (K62 shown in Figure 6), monocytes/macrophages lines and fibroblasts (not shown) were negative. Antibodies of the second type against the peptide of the putative intracellular stretch of m-MB-1 were negative for surface staining with all cell lines tested, i.e. with pre-B, B, plasmacytoma, T, monocyte/macrophage and fibroblast cells.

The second type of antibodies specific for the m-MB-1 peptide was also used for intracytoplasmic immunofluorescence staining, which was observed visually under the fluorescence microscope. Weak staining in sometimes spotty, sometimes pearl-chain-like forms, often on the rims of the cells, was observed with all pre-B and B cell lines tested (40E-1, 220-8, 204-3-1, 18-11, 70Z/3, WEHI 231, WEHI 279), but not with plasmacytomas (Sp2/0, MPC 11), nor with T cell hybridomas (A32-26, K62), monocyte/macrophages (WEHI 3), and fibroblasts (Ltk$^-$). Control antibodies not staining the cytoplasm of any type of cell were affinity-purified antibodies for peptides unrelated to m-MB-1.

These results suggest that specific antibodies of the first type detect the product of the m-*mb-1* gene on the surface of pre-B and B cell lines, while antibodies of the second type do so with the same types of cells in their cytoplasms. It is therefore likely that the amino-terminal domain of m-MB-1 is extracellular, while the carboxy-terminal domain could be intracellular.

Fig. 5. Sequencing strategies, nucleotide sequences, deduced amino acid sequences and Chou–Fasman prediction plots of hydrophilicity/hydrophobicity of the murine m-*mb-1*-W-8 clone (m), and the partial sequence of a human cDNA clone (h). The insert DNA fragments of both clones were analysed by restriction mapping and the restriction fragments were subcloned in M13 and mp18 and mp19 vector. Both strands were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) as in a. The arrows indicate the start, the direction and the extent of sequencing for the murine cDNA clone. Numbers shown above the amino acid sequence designate amino acid sequence position, while numbers beside the nucleotide sequence indicate the nucleotide position starting from the ATG initiation codon in the longest open coding frame of the murine clone. This is also borne out in the Chou–Fasman plot (Chou and Fasman, 1978) where this putative transmembrane region as well as the leader sequence show as clusters of hydrophobic amino acids. In the Chou–Fasman plot hydrophilicity of > -1.3 shows as ◊, hydrophobicity of > -1.3 as ◊, the two putative carbohydrate attachment sites as O, and cysteines as ■. Poly(A) attachment consensus sequences are underlined. ● are putative carbohydrate attachment sites, ○ signifies a charged amino acid within the hydrophobic stretch of amino acids, which may be a transmembrane portion of the m-MB-1 protein. Boxed amino acids are those synthesized for peptide-specific antibodies (see Materials and methods, and the text).

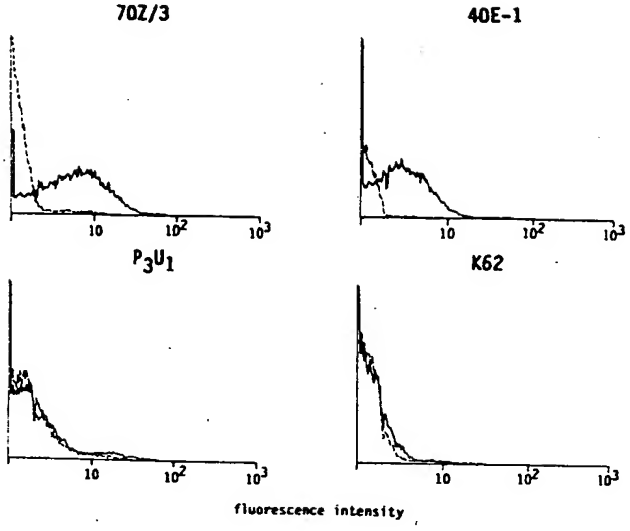

Fig. 6. Expression of m-*mb-1*-encoded protein sequences on different types of cells. Flow cytometric histograms are shown for 70Z/3 and the 40E-1 pre-B, P_3U plasmacytoma and K62 T hybridoma cell lines. Cells were stained with (——) or without (---) m-MB-1/protein fusion protein Ig-specific rat monoclonal antibody 1-20. The secondary reagent was FITC-conjugated goat anti-rat Ig antibody.

The two types of antibodies were also used with human cells, i.e. with Nalm-6, a human B lineage tumour cell line, and with Jurkat, a human thymoma line. The monoclonal antibody against the fusion protein of m-MB-1/protein A did not stain the surface of the two cell lines. The rabbit antibodies specific for the putative intracytoplasmic peptide of m-MB-1 did stain the cytoplasm of the Nalm-6 B lineage, but not the cytoplasm of the Jurkat T lineage cells. Staining was again spotty, with pearl-chain-like structures often accumulated on the rim. These results suggest that the peptide-specific antibodies can also detect the product of the human homologue of m-*mb-1*, again in intracytoplasmic spaces.

Discussion

After the discovery of λ_5, $V_{preB}1$ and $V_{preB}2$ (Sakaguchi and Melchers, 1986; Kudo *et al.*, 1987; Kudo and Sakaguchi, 1987) m-*mb-1* is the fourth gene which we have isolated from a pre-B cell-specific cDNA library which is selectively expressed in the B lymphocyte lineage. We have taken a series of transformed or malignant cell lines as representatives of normal states of cell differentiation to indicate this lineage-specific expression. We also demonstrate that normal resting B cells as well as polyclonally-activated, dividing B blasts, as examples of normal cells, express m-*mb-1*, in contrast to the other three B lineage-specific genes which are turned off when pre-B cells become mature B cells. It remains to be shown that normal pre-B cells express m-*mb-1*, although the 5–15% cells of fetal liver which were stained with a radiolabelled probe by *in situ* hybridization are an indication that normal pre-B cells could express the gene.

Stimulation of resting B cells into cell cycle by LPS appears to upregulate the expression of m-*mb-1*. This raises the question whether normal plasma cells do or do not express the gene, since plasmacytomas have been found not to express the gene. The activated B cell blasts which we have investigated for m-*mb-1* expression do secrete Ig, but are morphologically blast cells, rather than plasma cells, and have previously been shown to be capable of continued proliferation (Andersson *et al.*, 1977). Fully mature plasma cells may never develop in the 5–10 days of tissue culture in which activated B cells remain alive after LPS stimulation. We have not yet been able to isolate in purified form a fraction of non-dividing, mature plasma cells *ex vivo* which could be assayed for m-*mb-1* expression. It therefore remains a speculation that m-*mb-1* expression is turned off, as proliferating B cell blasts mature to non-proliferating plasma cells.

m-*mb-1* appears to be expressed as a protein on the surface of pre-B and mature B cell lines. This is suggested by the immunofluorescence staining of the cells with antibodies specific for a m-*mb-1*/protein A fusion protein, or specific for a m-*mb-1*-encoded peptide. From the staining pattern with the latter antibodies we suggest that the putative intracellular domain of m-MB-1 is, in fact, intracellular, and approachable by the specific antibodies after cytocentrifugation and fixation of the cells. Unfortunately, both types of antibodies have so far not specifically immunoprecipitated a protein of a mol. wt predicted from the deduced amino acid sequence, including the two potential carbohydrate attachment sites, from either internally ([^{35}S]methionine)- or externally (lactoperoxidase + ^{125}I)-labelled cells. The background of precipitated, unspecific bands from either Nonidet P-40- or digitonin-lysed cells is far too high to detect specific protein bands with certainty.

Although the nucleotide and deduced amino acid sequence of m-*mb-1* show no detectable similarity or homology to any known gene or protein, the overall structural features of m-MB-1 as a membrane-bound glycoprotein, with a predicted mol. wt of the mature protein of 20 kd, plus a leader peptide of 2 kd, and additional carbohydrate which might result in a glycoprotein of 26–35 kb, with an extracytoplasmic, a transmembrane, and an intracytoplasmic domain, makes m-MB-1 look very similar to the γ-chain of the CD3 complex expressed in association with the antigen-binding α/β or γ/δ chains of the T cell receptor on T cells (Clevers *et al.*, 1988). The very high homology of the nucleotide and amino acid sequences of murine and human *mb-1* is again very similar to the high homology of murine and human CD3 γ chains (van den Elsen *et al.*, 1984, 1985; Krissansen *et al.*, 1987) and might indicate a preserved function of the intracytoplasmic domain which, in the case of CD3 γ chains, has been suggested to be connected to signalling during activation of the cell. In analogy to CD3, where γ and δ chains show strong sequence homologies, one should even be prepared to expect other genes with high homology to m-*mb-1* to be expressed in the B lineage.

m-MB-1 might be related to the 28-kd protein which is co-precipitated with surface Ig by Ig-specific antibodies, and which appears to reside in the lipid bilayer of the surface membrane of B cells (Sidman *et al.*, 1980). In the human, the closest counterpart of m-MB-1 could be the surface antigen B1 (CD20) (Stashenko *et al.*, 1980; Foon and Todd, 1986) which has been reported to be 35 kd in size (Oettgen *et al.*, 1983). Since the general structure of m-MB-1 resembles that of the γ chains of the CD3 complex on T cells, it is tempting to speculate that a CD3-like complex of membrane-bound proteins could be associated with surface Ig on B cells, and might function to signal B cells for proliferation and maturation. At the moment, however, we have no evidence for an association of m-MB-1 with surface

Ig. As much as we are unable to identify the m-MB-1 protein by specific immunoprecipitation we have also failed to detect m-MB-1 associated with Ig chains immunoprecipitated by Ig-H or L-chain specific antibodies from Nonidet P-40 or digitonin lysates of cells. Furthermore, the m-MB-1-specific antibodies have so far not been found to be active in B cell activation, as CD3-specific antibodies were found with T cells (Meuer *et al.*, 1984). Generation of more antibodies, possibly against other determinants of m-MB-1, and a refinement of the biochemical identification of membrane proteins with affinities for Ig chains by specific serological techniques should clarify whether m-MB-1 is or is not a member of a CD3-like complex on B cells. Apart from its possible function, m-*mb-1* remains a useful marker gene for the B lineage in mice and humans.

Materials and methods

Mice

(C57BL/6J × DBA/2J)F_1 mice, 6–8 weeks old, and timed pregnancies of female C57BL/6J mice, mated with male DBA/2J mice induced by putting male and female mice together overnight, were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland. Day 1 gestation was counted as the day after mating. Birth occurred on day 19.

Cells, cell lines and culture conditions

Fetal liver cells from timed pregnant mice were prepared as described (Melchers *et al.*, 1977). Spleen cells, bone marrow cells and thymus cells were prepared as cell suspensions. To further enrich splenic B cells from other cells, cell suspensions were first treated with the monoclonal Thy-1-specific antibody J-1-j (obtained from Dr J.Sprent, Scripps Clinic and Research Foundation, La Jolla, CA) plus rabbit complement to deplete T cells. The T cell-depleted spleen cells were then further enriched for small resting cells by velocity sedimentation (Miller and Phillips, 1969). Small resting splenic B cells were stimulated by LPS (25 μg/ml) (S-form, kindly given to us by Drs C.Galanos and O.Lüderitz, Max Planck Institut für Immunobiologie, Freiburg i. Br., FRG) and 10% supernatant medium of 48 h LSP (2 μg/ml)-stimulated P388D1 cells as described (Corbel and Melchers, 1984). In order to obtain cell suspensions enriched for activated B cells they were subjected to velocity sedimentation after 3 days of activation (Melchers and Lernhardt, 1985). Cultures were done in serum-substituted medium (Iscove and Melchers, 1978). The virus-transformed pre-B cell Abelson lines, 18.81, 220-8, 204-1-8, were obtained from Dr John McKearn (now at Dupont Glenolden Laboratories, Glenolden, PA) and from Dr Naomi Rosenberg (Tufts University, Boston, MA). 70Z/3 and WEHI 231 cells were obtained from Dr Christopher Paige (Basel Institute for Immunology). The B cell lymphoma lines WEHI 279 (Sibley *et al.*, 1980), 2PK-3 and the thymoma EL 4 were given to us by Dr John McKearn. The macrophage lines P338D1 and WEHI 3 (D^-) were given to us by Dr N.Iscove (Ontario Cancer Research Institute, Toronto, Canada). The thymic lymphoma BW5147 ($HPRT^-$, Ova^n) was given to us by Dr Bob Hyman (Salk Institute for Biological Studies, La Jolla, CA).

The retrovirus-transformed early lymphoid progenitor cell line HAFTL-1 cells, its lymphoid sublines subclone 6, and its myeloid subclone 2B7 (Holmes *et al.*, 1986) were given to us by Drs Wendy Davidson and Jacalyn Pierce (National Institutes of Health, Bethesda, MD).

The other cell lines were X63, the Ig-nonproducing hybridoma, SP2/0 (given to us by Dr G.Köhler, Max Planck Institute for Immunobiology, Freiburg i. Br., FRG), MPC11 (given to us by Dr M.Scharff, Albert Einstein College of Medicine, Bronx, NY), the IgA-secreting plasmacytoma J558, the fibroblast cell line Ltk^- (given to us by Dr M.Burger, Biozentrum, University of Basel, Basel, Switzerland), and K62 T cell hybridoma line (given to us by Dr Anthonius Rolink, Basel Institute for Immunology). All cell lines and their known properties except for HAFTL-1 and its sublines were listed previously (Sakaguchi *et al.*, 1986). The human cell lines Nalm-6 and Jurkat were given to us by Dr Max D.Cooper (University of Alabama at Birmingham, AL).

All cell lines were grown in RPMH 1640 medium containing 2 mM glutamine, 5% heat-inactivated fetal calf serum (Gibco), β-mercaptoethanol (5×10^{-5} M), streptomycin (100 μg/ml) and penicillin (100 μg/ml) in a 10% CO_2 atmosphere.

Cloning for B cell-specific cDNA clones

A subtracted cDNA library for B cell-specific clones was constructed as described previously (Sakaguchi *et al.*, 1986). Briefly, the first cDNA strand from microsome-bound polysomal poly(A)$^+$ RNA of uninduced 70Z/3 cell line was enriched for B cell-specific sequences by repeated subtractive hybridization with excess amounts of T cell mRNA of the K62 hybridoma cell line. Double-stranded cDNA was synthesized after hydroxylapatite chromatography and ligated to λgt11 phage vector (Huynh *et al.*, 1985) with *Eco*RI linkers.

Two hundred individual recombinant clones were isolated by the differential hybridization of duplicate filters with radioactive cDNA probes from mRNA of either 70Z/3 or K62 cells. One clone, pZ176, positive on 70Z/3 and negative on K62 mRNA, was used for further screening.

A second cDNA library was later constructed from poly(A)$^+$ RNA of non-stimulated WEHI 231 B cell lymphoma by the method described (Gubler and Hoffman, 1983) with full-size inserts. Screening of 300 000 recombinant clones in λgt10 vector with the pZ176 gene probe yielded 62 positive clones. One of the positive clones containing a 1-kb insert, m-*mb-1*-W-8, was sequenced and used for the study of differential expression in the B lineage cells.

Isolation of human cDNA clones corresponding to murine m-mb-1

A cDNA library from poly(A)$^+$ mRNA of the human Burkitt lymphoma cell line was constructed as described above. Seven individual clones were isolated by screening of the library with the radiolabelled insert of the murine m-*mb-1*-W-8 clone. Final washing conditions of the filter hybridizations were 0.5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) 0.1% SDS at 60°C. One of the clones, named D-4, was used for sequence analysis.

Northern and Southern blot analysis

For Northern blot analysis, cytoplasmic RNA was prepared by the modified citric acid/phenol extraction method (Carmichael and McMaster, 1980). Briefly, $2-5 \times 10^8$ cells were homogenized in 30 ml of 5% citric acid solution and centrifuged to remove nuclei at 3000 *g* for 10 min. The supernatant was centrifuged at 10 000 *g* for 30 min at 4°C. The RNA pellet was dissolved in 20 ml of 0.2 M Tris–HCl pH 7.5, 5 mM EDTA, 2% SDS, 20 μl/ml heparin, homogenized again in a Dounce homogenizer and extracted twice with phenol:chloroform:isoamylalcohol (50:48:2). Poly(A)$^+$ RNA was prepared by oligo(dT)–cellulose column chromatography as described previously (Sakaguchi *et al.*, 1986). Five micrograms of poly(A)$^+$ RNA samples were electrophoresed by the agarose/formaldehyde method and transferred to nylon membrane as described (Maniatis *et al.*, 1982).

For Southern blot analysis high mol. wt genomic DNA was digested with restriction endonucleases and blotted as described by Southern (1975). Radiolabelling of insert DNA of the m-*mb-1*-W-8 clone, hybridization and washing conditions were as previously described (Sakaguchi *et al.*, 1986). Final blot washing conditions were 0.2 × SSC/0.1% SDS at 65°C (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

Subcloning and DNA sequencing

The 1-kb insert fragment of the m-*mb-1*-W-8 cDNA clone in λgt10 was subcloned in pUC-13. After restriction enzyme analysis, restriction fragments were further subcloned into M13mp18 and mp19 vectors using the appropriate enzyme sites. The 1-kb insert was sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977).

Preparation of fusion protein between protein A and the m-mb-1-encoded polypeptide

The protein A/m-*mb-1* expression plasmid was constructed by introducing cDNA insert from m-*mb-1*-W-8 clone into the *Eco*RI sites of pR1T2T plasmid DNA (Lowenadler *et al.*, 1986) in the appropriate frame and the appropriate orientation. The chimeric protein was purified as described (Lowenadler *et al.*, 1986) and was used as the antigen to immunize Lewis rats.

Production of hybridomas

B cell hybridomas between rat spleen cells activated with the fusion protein and the variant, drug-resistant myeloma cell line P3 × 63AG8.653 were produced by standard techniques (Köhler and Milstein, 1975) with polyethylene glycol as fusion agent, and hybrids selected in HAT medium. An ELISA assay, using plates coated with the fusion protein and excess human IgG to block protein A binding (Lowenadler *et al.*, 1986), was used to screen for specific monoclonal antibodies. A goat antibody with specificity for Ig coupled with alkaline phosphatase (BioRad Laboratories, Richmond, VA) was used as enzyme active agent in the ELISA.

Three independent hybridomas showed the same specificity of fluorescent staining pattern by flow cytometry analysis on various cell lines including pre-B cells, myeloma and T cells. One typical example is shown in Figure

6. This monoclonal antibody, 1-20, showed positive staining on pre-B cells 40E-1, 70Z/3, but not on myeloma cell P3U1 or on T cell hybridomas K62.

m-MB-1 peptide-specific antibodies

The peptide indicated in Figure 5 (amino acid positions 187–220) was synthesized with an Applied Biosystems peptide synthesizer according to the Merrifield solid-phase method on a 4-methylbenzhydrylamine resin (Merrifield, 1963; Steward *et al.*, 1976). The peptide was purified by HPLC on a preparative C_{18} reversed-phase column (Vydac) and analysed, after hydrolysis, in an Applied Biosystems automated amino acid analyser. (We thank Mr D.Avita and Dr J.D.Lambris of our Institute for performing the synthesis.) The peptide, containing at its amino-terminal end a cysteine residue, was coupled to ovalbumin by SPDP [*N*-succinimidyl-3-(2-pyridyldithio)-propionate, Pharmacia AB, Uppsala, Sweden].

Rabbits were immunized with the peptide–ovalbumin conjugate and the peptide-specific antibodies purified by absorption to Sepharose-coupled peptide [using 4 mg peptide to couple to 1 ml of packed CNBr-activated Sepharose (Pharmacia AB, Uppsala, Sweden)], followed by elution of the peptide-specific antibodies with 0.9% NaCl, 0.1 M glycine-HCl pH 2.2. The specific antibodies were tested on a panel of peptides of other sequences by ELISA using an alkaline phosphatase-coupled pig anti-rabbit antibody as developing agent, and were found to be specific for the m-MB-1 sequence.

Immunofluorescence analyses

The rat monoclonal antibodies, when bound to cells, were detected by FITC-conjugated goat anti-rat antibodies (Nordic Immunology, The Netherlands); rabbit antibodies were detected by FITC-labelled goat anti-rabbit Ig antibodies. Cells were stained for surface and for intracytoplasmic detection of determinants by described methods (Andersson *et al.*, 1974; Forni and de Petris, 1984).

$5 \times 10^5 - 1 \times 10^6$ cells in 80 μl phosphate-buffered saline (PBS), 1% fetal calf serum (FCS), 2 mM NaN_3 were incubated with 20 μl specific antibody (~20 μg/ml) for 30 min on ice. After washing twice in PBS, 1% FCS, 0.2 M NaN_3 50 μl FITC-labelled antibodies were added. Cells were incubated for 20 min on ice and subsequently washed twice in PBS, 1% FCS, 2 mM NaN_3. Propidium iodide (Sigma, St Louis, MO) was added at a final concentration of 5 μg/ml to exclude dead cells from analysis. Cells were analysed on a Becton Dickinson analyser (Becton Dickinson, Oxnard, CA) or a FACS IV equipped with an argon laser tuned to 488 nm, operating at 200 mW power. Fluorescence histograms were generated with logarithmic or linear amplification of fluorescence emitted by single viable cells. A minimum of 1×10^4 cells were analysed per sample. Cell surface antigen expression on cell lines was quantitated as relative fluorescence intensity in arbitrary units, using peak fluorescence channel measurements. All experiments with cell lines were repeated at least three times. Intracytoplasmic staining of cytocentrifuged, fixed cells was done with a Leitz orthoplan microscope equipped with an Opak-Fluor vertical illuminator (Leitz GmbH, Wetzlar, FRG).

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References

Andersson,J., Sjöberg,O. and Möller,G. (1972) *Eur. J. Immunol.*, **2**, 349–353.

Andersson,J., Buxbaum,J., Citronbaum,R., Douglas,S., Forni,L., Melchers,F., Pernis,B. and Stott,D. (1974) *J. Exp. Med.*, **140**, 742–763.

Andersson,J., Coutinho,A., Lernhardt,W. and Melchers,F. (1977) *Cell*, **10**, 27–34.

Berger,C.N. (1986) *EMBO J.*, **5**, 85–93.

Carmichael,G.G. and McMaster,G.K. (1980) *Methods Enzymol.*, **65**, 380–402.

Chou,P.Y. and Fasman,G.D. (1978) *Annu. Rev. Biochem.*, **47**, 251–276.

Clevers,H., Alarcon,B., Wileman,T. and Terhorst,C. (1988) *Annu. Rev. Immunol.*, **6**, 629–662.

Coffman,R.L. and Weissman,I.L. (1981) *Nature*, **289**, 681–683.

Cooper,M.D., Mulvaney,D., Coutinho,A. and Cazenave,P.A. (1986) *Nature*, **312**, 616–618.

Corbel,C. and Melchers,F. (1984) *Immunol. Rev.*, **78**, 51–74.

Foon,K.A. and Todd,R.F., III (1986) *Blood*, **68**, 1–31.

Forni,L. and de Petris,S. (1984) *Methods Enzymol.*, **108**, 413–425.

Gubler,U. and Hoffman,B.J. (1983) *Gene*, **25**, 263–269.

Holmes,K.L., Pierce,J.H., Davidson,W.F. and Morse,H.C., III (1986) *J. Exp. Med.*, **164**, 443–457.

Huynh,T., Young,R.A. and David,R.W. (1985) In Glover,D.M. (ed.), *DNA Cloning—A Practical Approach*. IRL Press, Oxford, Vol. 1, pp. 49–78.

Iscove,N.N. and Melchers,F. (1978) *J. Exp. Med.*, **147**, 923–933.

Kincade,P.W., Lee,G., Watanabe,T., Sun,L. and Schied,M.P. (1981) *J. Immunol.*, **127**, 2262–2266.

Köhler,G. and Milstein,C. (1975) *Nature*, **256**, 495–496.

Krissansen,G.W., Owen,M.J., Fink,R.J. and Crumptors,M.J. (1987) *J. Immunol.*, **138**, 3513–3518.

Kudo,A. and Melchers,F. (1987) *EMBO J.*, **6**, 2267–2272.

Kudo,A., Sakaguchi,N. and Melchers,F. (1987) *EMBO J.*, **6**, 103–107.

Lowenadler,B., Nilsson,B., Abrahmsen,L., Moks,T., Lundqvist,L., Holmgren,E., Paleus,S., Josephson,S., Philipson,L. and Uhlen,M. (1986) *EMBO J.*, **5**, 2293–2298.

Maniatis,T., Fritisch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

McKearn,J.P., Baum,C. and Davie,J.M. (1984) *J. Immunol.*, **132**, 332–333.

Melchers,F. and Lernhardt,W. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7681–7685.

Melchers,F., Andersson,J. and Phillips,R.A. (1977) *Cold Spring Harbor Symp. Quant. Biol.*, **41**, 147–158.

Merrifield,R.B. (1963) *J. Am. Chem. Soc.*, **85**, 2149.

Meuer,S.C., Hussey,R.E., Cantrell,D.A., Hodgdon,J.C., Schlossman,S.F., Smith,K.A. and Reinherz,E.L. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1509–1513.

Miller,R.G. and Phillips,R.A. (1969) *J. Cell. Physiol.*, **73**, 191–202.

Oettgen,H.C., Bayard,P.J., Van Ewijk,W., Nadler,L.M. and Terhorst,C.P. (1983) *Hybridoma*, **1**, 17–28.

Sakaguchi,N. and Melchers,F. (1986) *Nature*, **324**, 579–582.

Sakaguchi,N., Berger,C.N. and Melchers,F. (1986) *EMBO J.*, **5**, 2139–2147.

Sanger,F., Nicklen,S. and Coulson,A. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Sibley,C.H., Ewald,S.J., Kehry,M.R., Douglas,R.H., Raschke,W.C. and Hood,L.E. (1980) *J. Immunol.*, **125**, 2097–2105.

Sidman,C.L., Bercovici,T. and Gitler,C. (1980) *Mol. Immunol.*, **17**, 1575–1583.

Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.

Stashenko,P., Nadler,L.M., Hardy,R. and Schlossman,S.F. (1980) *J. Immunol.*, **125**, 1678–1685.

Stewart,J., Pensa,C., Matsueda,G. and Harris,K. (1976) In Loffer,A. (ed.) *Peptides*. Editions de l'Université de Bruxelles, Bruxelles, pp. 285–290.

Tonegawa,S. (1983) *Nature*, **302**, 575–581.

Van den Elsen,P., Shepley,B.A., Borst,J., Coligan,J., Markham,A.F., Orkin,S. and Terhorst,C. (1984) *Nature*, **312**, 413–418.

Van den Elsen,P., Shepley,B.A., Cho,M. and Terhorst,C. (1985) *Nature*, **314**, 542–544.

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